

## Monitoring Multiple Pluripotency Biomarkers After Delivery of Reprogramming Factors to Human Somatic Cells

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### Summary

Trans-acting factors present in the mammalian oocyte are capable of reprogramming somatic cell nuclei to an undifferentiated state and produce clonal embryonic stem cells (ESCs) in a process called somatic cell nuclear transfer. Recent studies demonstrate that a basic set of transcription factors (Oct4, Sox2, cMyc and Klf4) are sufficient to reprogram mouse somatic cells to pluripotent stem cells that exhibit the essential characteristics of ES cells. It is anticipated that such induced pluripotent stem (iPS) cells will be useful for development of disease models, drug studies and eventually patient-specific tissue replacement. This investigation demonstrates that expression of these four factors, plus the additional transcription factor Nanog, from recombinant lentivirus, effectively reprogrammed human somatic cells such that they displayed a pluripotent phenotype and expressed multiple pluripotency genes. It was further demonstrated that the genes for these transcription factors were transiently transduced into human somatic cells with non-viral, particle vectors and transiently induced expression of pluripotency markers.

### Introduction

ESCs are unique in their ability to grow indefinitely while maintaining pluripotency. Their ability to differentiate into virtually any cell type makes them prime candidates for

regenerative medicine. However, ethical concerns surround the origins of human ESCs and practical concerns relate to the potential for immune rejection. It has been reported that pluripotent stem cells can be induced from mouse and human adult fibroblasts by introducing four transcription factors, Oct4, Sox2, cMyc, and Klf4, under ESC culture-conditions using retroviral vectors.<sup>1,2</sup> Such iPS cells have great potential to support regenerative and developmental research in conjunction with human ESCs.<sup>3,4</sup> It is thought that both could ultimately lead to personalized cell replacement therapies.<sup>5</sup> However, the use of retroviral vectors in a clinical environment is likely to be problematic due to the increased risk of tumorigenicity with genome-integrating viruses. Therefore, non-viral delivery mechanisms are highly desirable. This investigation sought to determine whether various types of human somatic cells could effectively be reprogrammed in the presence of five transcription factor genes (Oct4, Sox2, cMyc, Klf4 and Nanog) delivered either by lentivirus vectors or non-viral particles. Embryonic fibroblasts, adult retinal pigment epithelial cells and adult testicular cells were tested for their ability to de-differentiate and acquire ESC-like properties. The level of reprogramming was determined by cellular morphology, presence of surface markers and ultimately, the expression of pluripotency marker genes.

## Results

### *Morphology and cell surface marker analysis of transduced human somatic cells*

Human embryonic fibroblasts (HEF) were infected with recombinant lentivirus containing the cDNAs for five transcription factors (Oct4, Sox2, cMyc, Klf4 and Nanog) to verify that previously reported results<sup>1,2</sup> could be replicated in this study. Additionally, human adult retinal pigment epithelial (RPE) cells were also infected with the same combination of lentiviruses to determine if adult human somatic cells of a different tissue origin could be effectively reprogrammed. Colonies resembling stem cell colonies were detected after 14 (RPE) or 19 (HEF) days (Figure 1). Twenty of the RPE and three of the HEF colonies were successfully expanded. While the RPE colonies could be treated with trypsin at an early stage in expansion, the HEF colonies had to be picked manually. During expansion, 10% of the RPE colonies and 80% of the HEF colonies developed fibroblastic morphology. Taken together, these suggest a more sustained and robust change in the RPE colonies as compared to HEF. Cell surface marker analysis of the colonies revealed that the RPE cells did not stain for any embryonic stem cell surface marker, whereas the HEF colonies stained positive for the hESC surface markers SSEA4, Tra1-81 and Tra1-60 (Figure 1).

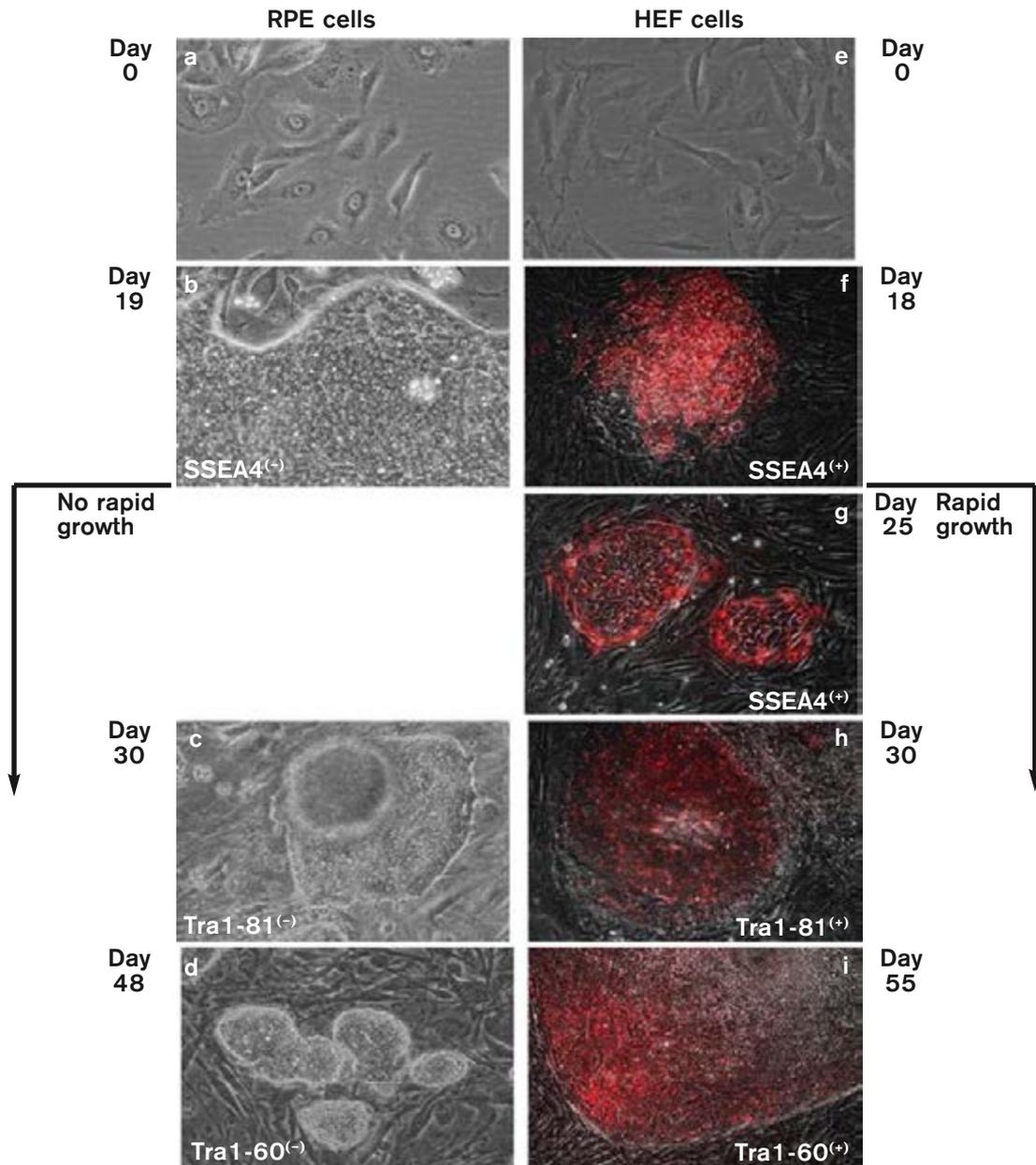
### *Gene expression analysis of transformed cells*

Multiplex gene expression profiling was used to measure the relative expression of each pluripotency marker in experimental cells. HEFs did not show any expression of pluripotent markers before infection (Figure 2). Expression of the five transduced transcription factors was first detected at seven days post-infection, accompanied by a slight upregulation of the fibroblast markers Thy1 and Col5A2, the mesoderm marker Brachyury and the germcell-specific marker cRET. Earlier reports of reprogramming in adult mouse tail fibroblasts suggested activation of ALPL and a downregulation of Thy1 as well as other fibroblast differentiation marker genes.<sup>6,7,8</sup> In this experiment however, the steepest increase of ALPL was detected after day 17. Moreover, the fibroblast marker genes Thy1 and Col5A2

increased markedly at seven days post-infection, but then decreased to the control level of expression seen in untreated HEFs. These results suggest that the timeline and marker expression profiles in human and mouse fibroblasts may be different during reprogramming.

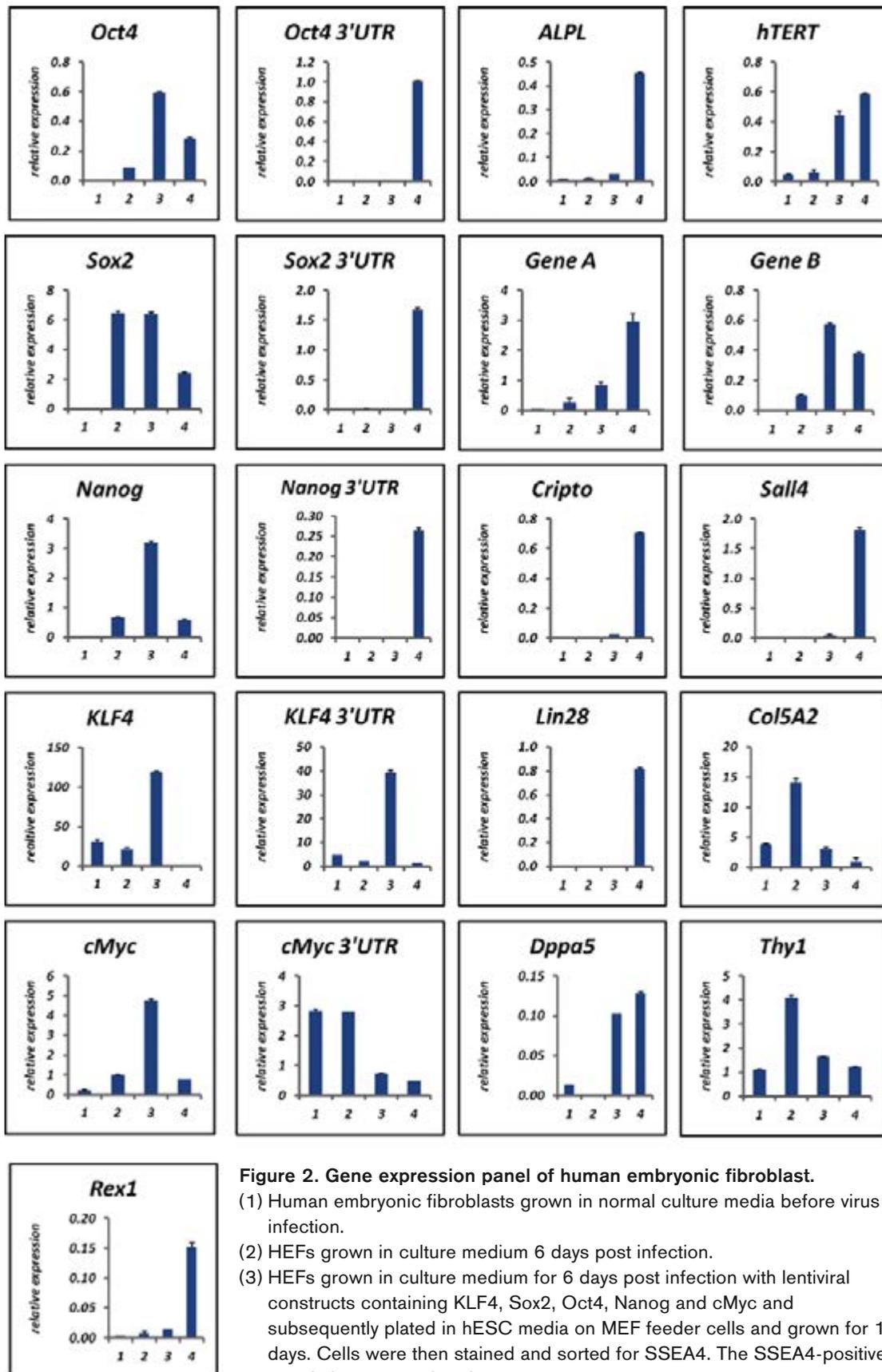
The levels of cDNA expression of pluripotent genes in the SSEA4<sup>+</sup> population of HEFs at day 17 were greater than in the overall cell population at day seven (data not shown), suggesting a progressive enrichment of the virus-expressing cells in the SSEA4<sup>+</sup> population. There was small but distinguishable upregulation of a number of pluripotent marker genes (hTERT, ALPL, Cripto, Sall4 and Dppa5), suggesting that within the virus-expressing SSEA4<sup>+</sup> cells, only a small fraction of cells were de-differentiated, as described by Mikkelsen et al.<sup>8</sup> An alternative explanation would be the beginning of a global expression change leading to iPS cells. Nevertheless, after replating the SSEA4<sup>+</sup> population, no colonies could be detected. In summary, HEF colonies expressed a complete set of endogenous pluripotent marker genes 30 days post-infection, strongly implying that these cells were de-differentiated. Further studies are necessary to determine if these cells have full pluripotentiality.

Control RPE cells infected with GFP-lentivirus had low endogenous cMyc and intermediate levels of Klf4 expression, but no expression of any other pluripotent markers (Figure 3). After transduction and clonal expansion, one RPE clone (no. 6), had expression of Klf4, cMyc and Nanog, but lacked Oct4 and Sox2 cDNA expression. This strongly suggests that just two transcription factors, cMyc and Nanog, were transduced into these cells. For RPE colonies, endogenous Klf4 expression rose after 5-factor infection as did total (endogenous plus exogenous) Klf4 cDNA expression. However, this phenomenon was also observed in control RPE cells plated onto MEF feeder cells and grown in hESC medium (data not shown). It can therefore not be assumed that these RPE cells have been infected with the Klf4 virus, unless the cells have been tested for the viral insert. In summary, no upregulation of any of the tested pluripotent marker genes was



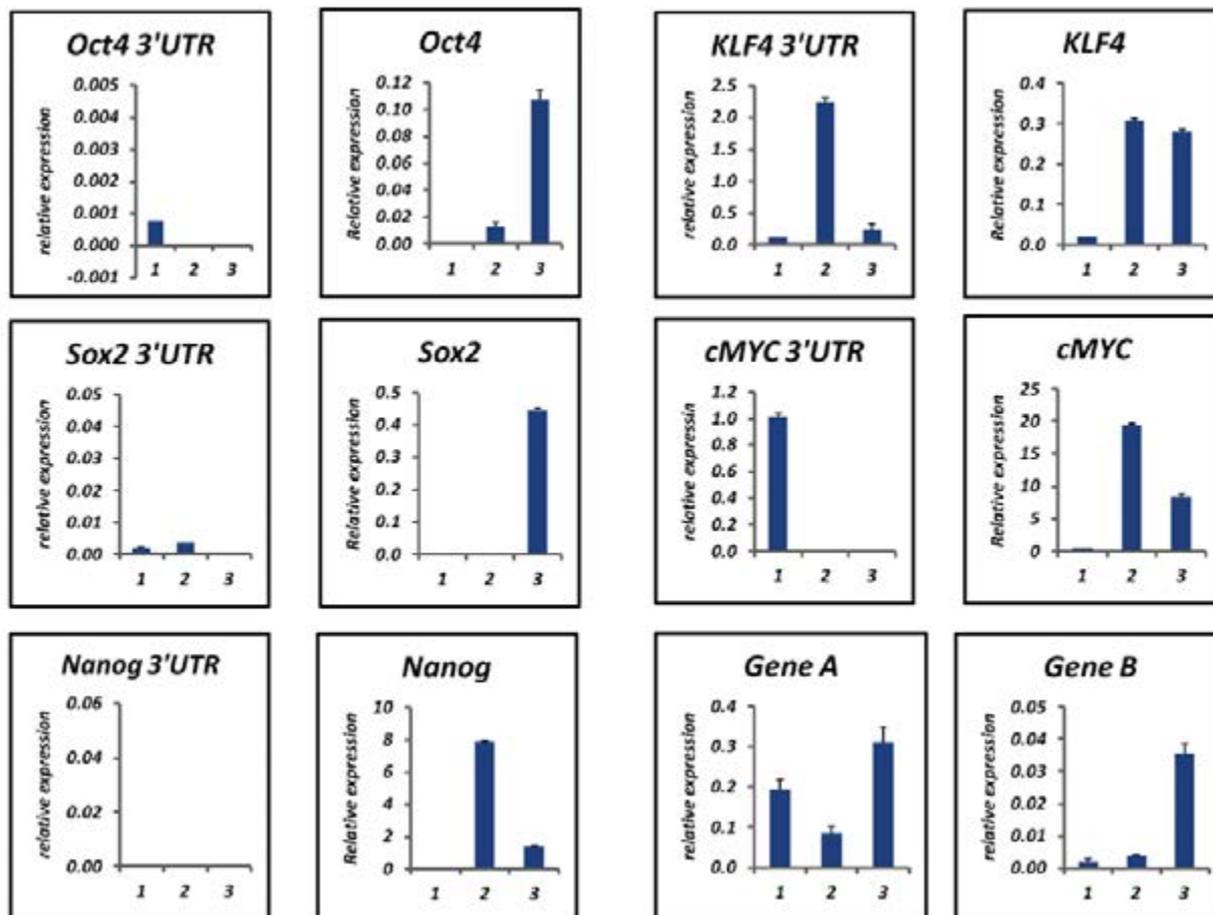
**Figure 1. Derivation of intermediate retinal pigment epithelial cells and human embryonic fibroblast iPS-like cells.**

- (a) RPE cells grown in normal media before virus infection.
- (b) RPE cells 18 days post infection with lentivirus containing Oct4, Sox2, KLF4, cMyc and Nanog virus. Cells have been grown in RPE culture medium for 6 days on a normal culture dish and subsequently seeded onto MMC-treated MEF feeder cells at a density of  $5 \times 10^4$  cells. Colonies emerged after 14 – 18 days with a frequency of approximately 1/500. Colonies did not stain for SSEA4.
- (c) The colonies could be picked and passaged onto new feeder cells by manual picking. The resulting colonies maintained their morphology, but grew slowly. Colonies did not stain for Tra1-81.
- (d) RPE colonies were trypsinized and passaged onto new feeder cells without losing their morphology. Colonies did not stain for Tra1-60.
- (e) Human embryonic fibroblasts grown before virus infection. (f) Six days post infection, infected HEF cells were seeded onto MEF feeder cells at a density of  $5 \times 10^4$  cells. HEF colonies emerging 18 days post infection at a rate of 1 in  $1 \times 10^4$ . Colonies staining for SSEA4 were manually picked and passaged onto fresh feeder cells.
- (g) Colonies grew rapidly and maintained their expression of SSEA4. (h) Resulting colonies also stained for Tra1-81 and (i) Tra1-60. The colonies maintained their morphology at a rate of 20%. Differentiated cells were observed at the shape and size of fibroblasts.



**Figure 2. Gene expression panel of human embryonic fibroblast.**

- (1) Human embryonic fibroblasts grown in normal culture media before virus infection.
- (2) HEFs grown in culture medium 6 days post infection.
- (3) HEFs grown in culture medium for 6 days post infection with lentiviral constructs containing KLF4, Sox2, Oct4, Nanog and cMyc and subsequently plated in hESC media on MEF feeder cells and grown for 11 days. Cells were then stained and sorted for SSEA4. The SSEA4-positive population was analyzed.
- (4) Established HEF iPS cell culture at day 30 post infection. Gene A is cRET and Gene B is Brachyury.



**Figure 3. Gene expression panel of retinal pigment epithelial cells.**

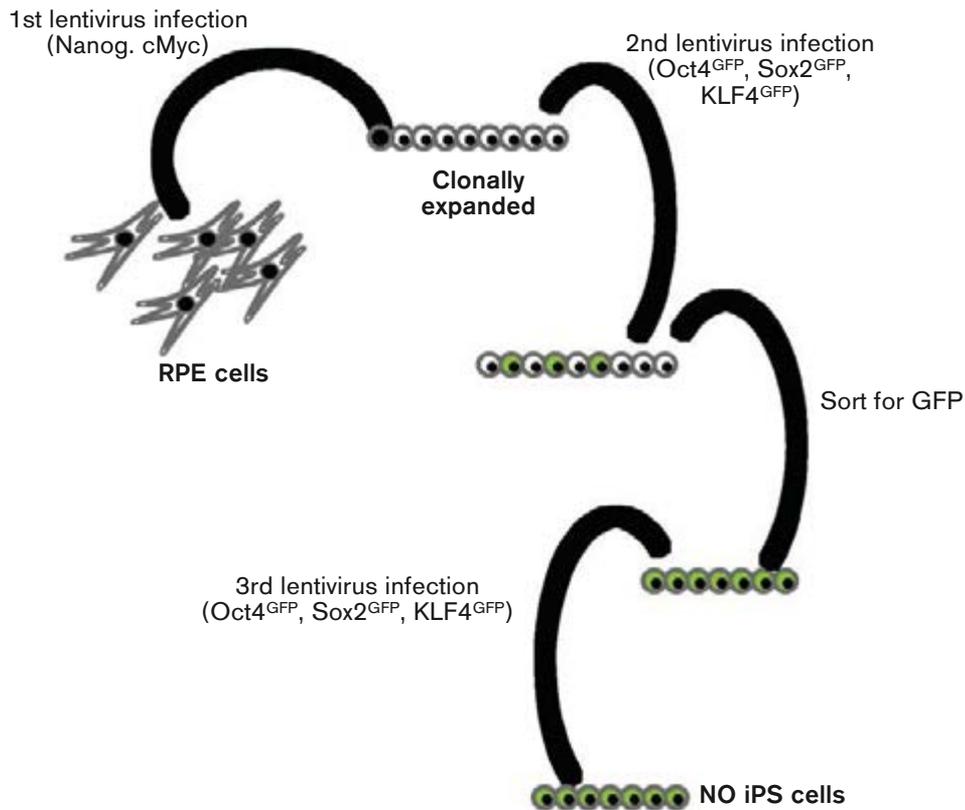
- (1) RPE cells grown in normal media before virus infection.
- (2) RPE cells grown on mitomycin C (MMC) treated mouse embryonic fibroblast (MEF) feeder cells in hESC media at 30 days post-infection with lentiviruses containing cDNAs encoding Oct4, Sox2, KLF4, cMyc and Nanog transcription factors.
- (3) RPE cells grown on MMC treated MEF feeder cells after two more rounds of subsequent virus.

observed 30 days post-infection in any of the resultant RPE cell lines.

In the interest of deriving iPS cells from these intermediate RPE cells, we tested whether the missing pluripotent transcription factors could be transduced at a later time point. For these experiments, RPE colonies were transduced with lentiviruses having a bi-cistronic construct containing Klf4, Oct4 or Sox2 in combination with GFP (Figure 4). Using this approach, it was possible to infect the RPE colonies, sort for GFP-positive cells to ensure that only infected cells were plated, and then infect again, thereby increasing the probability that all three transcription factors entered the cell. Gene expression analysis (Figure 3) showed that re-infection led to the uptake of Oct4, Sox2 and Klf4 cDNA. However, there was no

change in SSEA4-staining (data not shown) or endogenous pluripotent marker expression (Figure 3), suggesting that sequential infection under these conditions did not lead to an iPS state in the observed timeframe of 30 days. Similarly, it has been reported that ectopic expression of Oct4 and cMyc is sufficient to induce hESC-like morphological changes in human fibroblasts without inducing pluripotency.<sup>9</sup>

Interestingly, cRET and Brachyury are up-regulated early in the reprogramming process in HEF iPS-like cells (Figure 2), while there is little to no activation of those genes after initial infection of RPE cells (Figure 3). Only after re-infection of the Nanog- and cMyc-expressing RPE cells with the missing transcription factors is there a minor increase in the expression of



**Figure 4.** Flow chart for re-infection experiment.

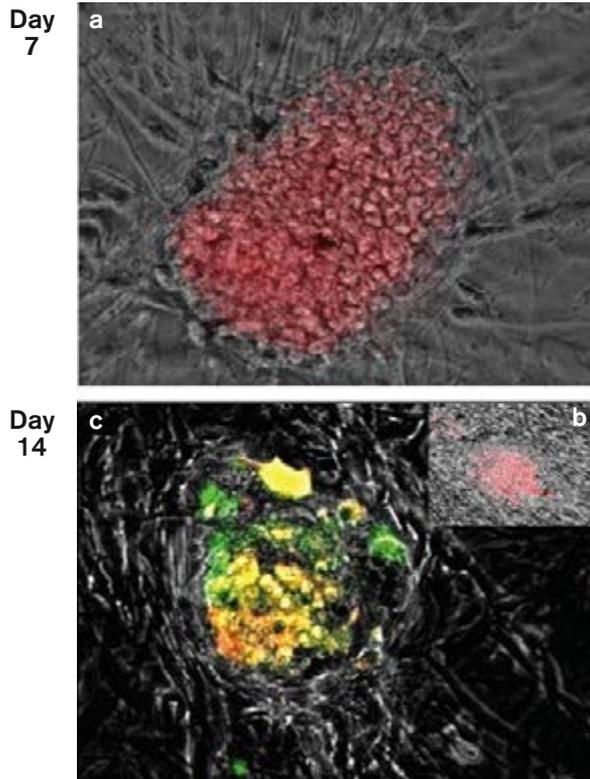
cRET and activation of Brachyury. One could speculate that the delayed activation in expression of Brachyury and cRET may be the cause for the failed de-differentiation in RPE cells.

#### *Non-viral delivery of transcription factor genes*

Non-viral genetic delivery mechanisms are highly desirable to avoid the drawbacks of viral vectors in the clinical setting. Single walled carbon nanotubes (SWNT) have been reported to bind and deliver DNA into the nuclei of cells.<sup>10</sup> The five transcription factor plasmid DNAs (Oct4, Sox2, Klf4, cMyc and Nanog) were bound to SWNT. Human testicular fibroblasts were stably transformed with a lentivirus encoding a red fluorescent protein (RFP) driven by the Nanog promoter to create RFP reporter cells (HT42 NP). Detection of RFP production served as a visual indicator of Nanog promoter activation and the initiation of cellular reprogramming. HT42 NP cells were incubated with the plasmid-bound SWNT particles. The transfection efficiency achieved with this method was consistently below 5% as determined by flow cytometry analysis (data not

shown). However, five days after transfection, a few RFP-positive colonies could be observed (Figure 5). These colonies grew slowly until day 12 and then stopped, still expressing RFP. While no cell lines resulted, it was possible to stain two RFP-positive colonies with anti-SSEA4 antibody (Figure 5). The results suggested that after transfection with 5 transcription factor plasmid DNAs, HT42 NP cells may enter a transient state of activation of both SSEA4 and the Nanog promoter. However, the failure to establish cell lines suggested that the levels of transcription factor proteins, or the timing of expression, were insufficient to allow stable reprogramming. Published results with a doxycycline-inducible retrovirus<sup>6,7,8</sup> suggest the necessity of stable transcription factor expression over at least nine days in mouse cells. Therefore, the expression profile of HT42 NP cells was evaluated after delivery of the five transcription factor plasmid DNAs coupled to SWNT. RNA samples were collected at Day 0 (8 hours), 7 and 14 days after treatment and expression was analyzed using multiplex XP-PCR.<sup>b</sup> Expression of plasmid DNAs introduced by SWNT was confirmed by XP-PCR and, as

## 5 TxFactor DNA



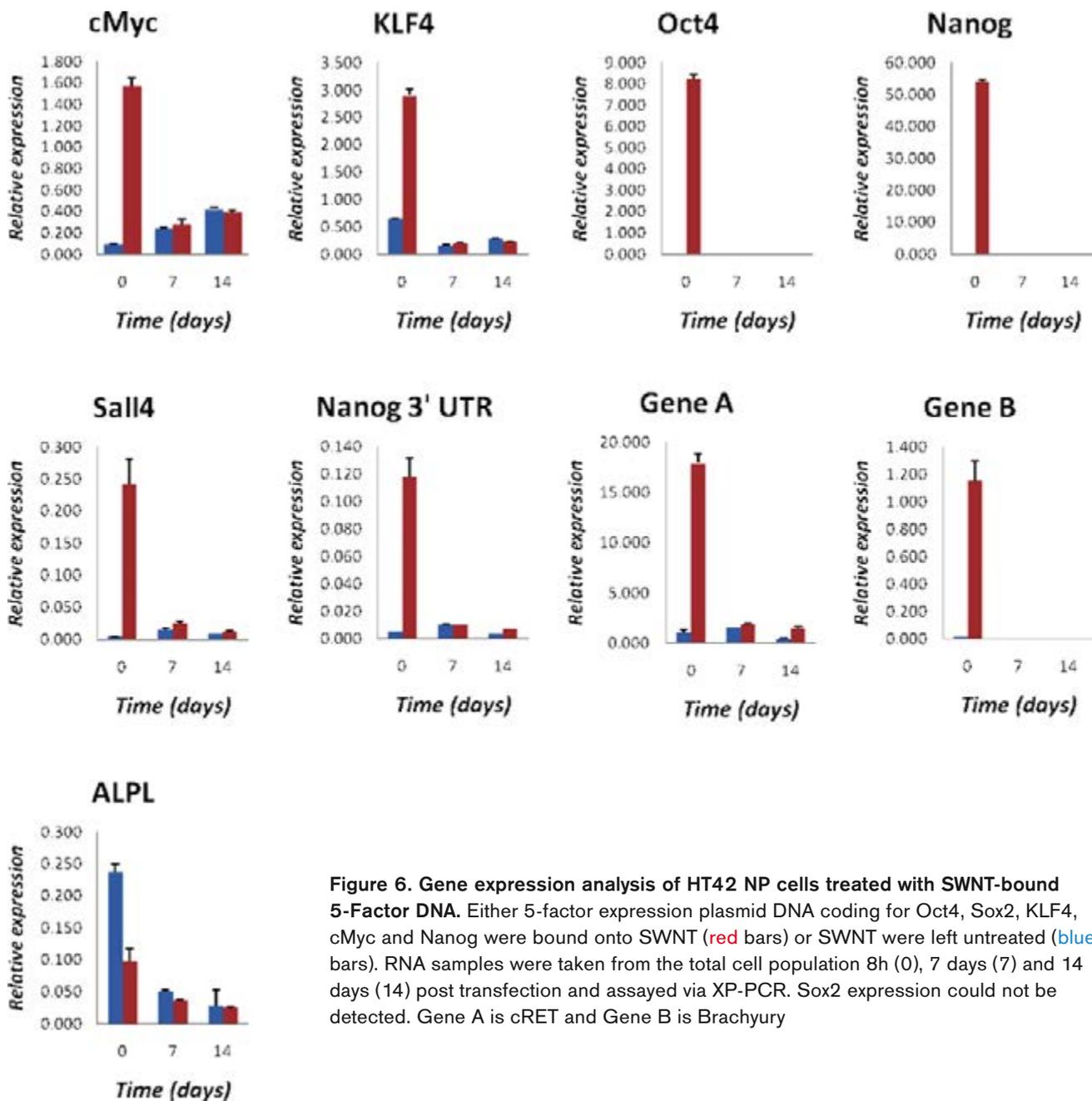
**Figure 5. SWNT-bound 5 transcription factor DNA leads to colony formation without establishing a cell line.**

- (a) HT42 NP cells 7 days after transfection with SWNT bound 5 Factor DNA. RFP expression is driven by the Nanog promoter.
- (b) HT42 NP cells 14 days after transfection with SWNT bound 5 Factor DNA. RFP expression is driven by the Nanog promoter.
- (c) HT42 NP cells 14 days after transfection with SWNT bound 5 Factor DNA. RFP expression is driven by the Nanog promoter. Colonies were stained with a FITC coupled SSEA4 antibody. A merge of the picture lead to yellow color in Nanog and SSEA4<sup>+</sup> cells. There was no further growth detectable after day 12 of transfection. All pictures are 20 x magnifications.

expected, the expression levels drastically decreased from day zero to day seven (Figure 6). Interestingly, expression levels of two pluripotent marker genes in the panel, *Sall4* and endogenous *Nanog* (*Nanog* 3'UTR), increased coincident with the expression from the plasmid DNA. Thus, a transient activation of the *Nanog* promoter in HT42 NP cells was confirmed by two different methods. Surprisingly, expression levels of the reported early reprogramming marker *ALPL*<sup>6,7,8</sup> were decreased relative to controls. This could be due to the fact that HT42 NP cells already have a high level of *ALPL* expression and in this case, the expected increase in *ALPL* may not have been detectable against the high background. In further support of the induced changes in human testicular (HT) cells after delivery of five transcription factors DNAs, *cRET* and *Brachyury* were consistently upregulated. Combined with similar findings in virally-transduced HEF cells, there is additional support for the idea that HT cells are responsive to reprogramming signals, but either the timing and/or co-expressed levels of transduced genes are not effective to induce a stable expression of pluripotent genes. Alternatively, *Sox2* expression could

not be detected in the treated HT cells and this could account for the observed defective reprogramming.

To investigate whether the failure to reprogram might be related to the type of delivery particle, HEFs were treated with the stable aggregates formed between the five transcription factor plasmid DNAs and polyethylene imine (PEI) particles. The latter delivery method consistently produced transfection efficiencies >25% (data not shown), in comparison to less than 5% with SWNT. The expression levels of the five transcription factors increased for 24 hours after infection and then dropped after 72 hours (Figure 7). In contrast to the SWNT treatment of HT42 NP cells, no change was observed in expression of *Sall4* or endogenous *Nanog* in HEF. Additionally, no colonies were observed with HEF (data not shown). The differences in pluripotent marker expression between HT and HEF imply that perhaps a more rapid activation of *Nanog* may occur in the HT cells than in HEFs, which may account for the presence of HT colonies and absence of HEF colonies with particle-based transduction. It is possible that intrinsic epigenetic genomic plasticity may play a role



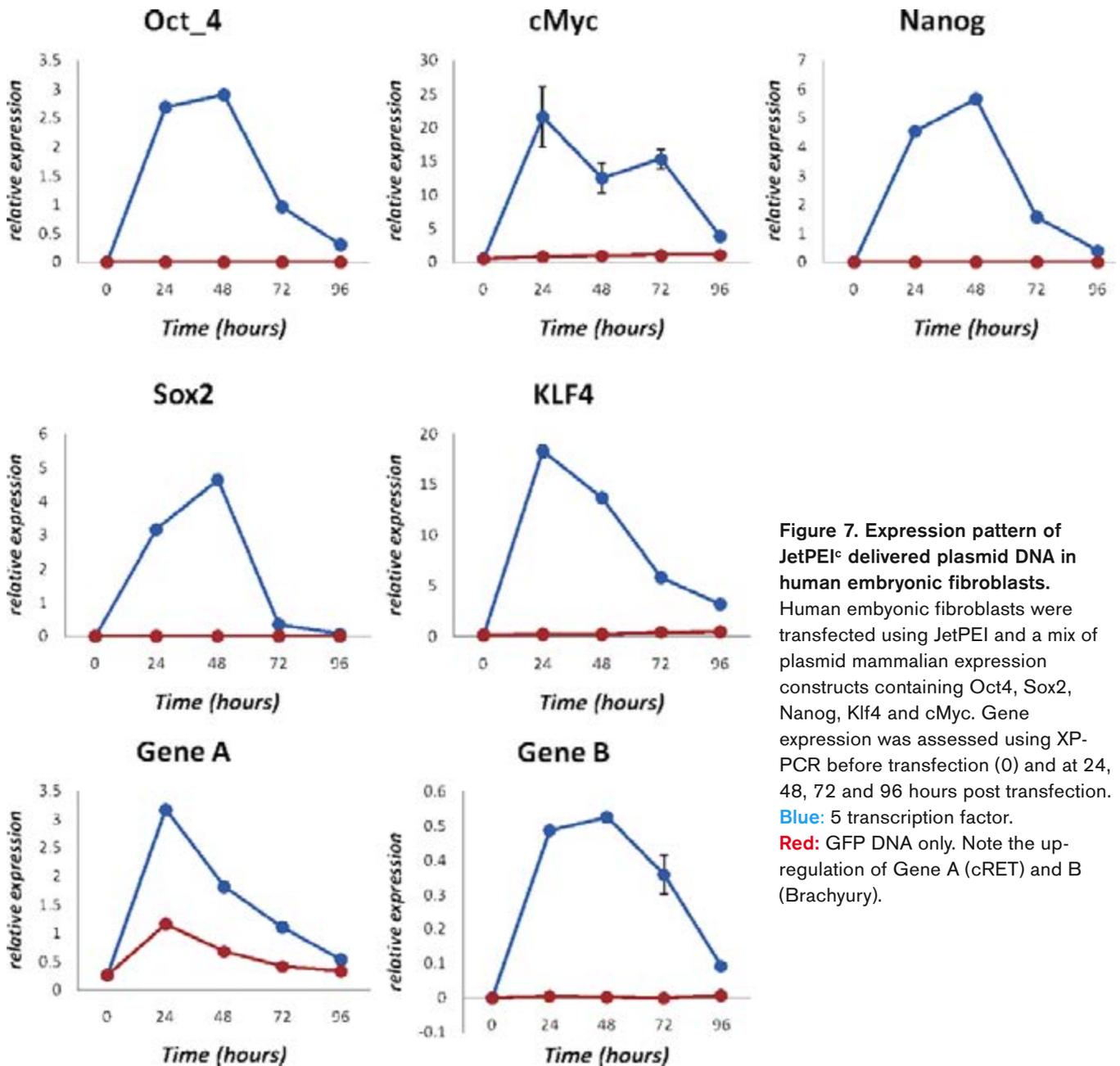
**Figure 6. Gene expression analysis of HT42 NP cells treated with SWNT-bound 5-Factor DNA.** Either 5-factor expression plasmid DNA coding for Oct4, Sox2, KLF4, cMyc and Nanog were bound onto SWNT (red bars) or SWNT were left untreated (blue bars). RNA samples were taken from the total cell population 8h (0), 7 days (7) and 14 days (14) post transfection and assayed via XP-PCR. Sox2 expression could not be detected. Gene A is cRET and Gene B is Brachyury

determining the potentiality of different cell types for iPS cell reprogramming. Additionally, the same two genes (cRET and Brachyury) that were upregulated in the HT experiments above were also transiently activated in HEFs (Figures 6 and 7, respectively), suggesting that these genes may be early downstream targets of one or a combination of the transduced cDNAs during the reprogramming process.

## Discussion

The ability to generate iPS cells from patient somatic cells promises regenerative therapy

without the need for immunosuppressive drugs to prevent unmatched cell rejection. However technical and safety obstacles must be overcome before this promise becomes reality. Previous work demonstrated the ability to create iPS cells from both embryonic and adult fibroblasts employing retroviral vectors.<sup>12</sup> In this study, we demonstrate the effective delivery of pluripotency genes in both adult human fibroblasts as well as retinal epithelial cells using retroviral vectors. ESC-like colonies were derived from human embryonic fibroblasts (HEF) that were infected with recombinant lentiviruses containing the coding



**Figure 7. Expression pattern of JetPEI<sup>®</sup> delivered plasmid DNA in human embryonic fibroblasts.** Human embryonic fibroblasts were transfected using JetPEI and a mix of plasmid mammalian expression constructs containing Oct4, Sox2, Nanog, Klf4 and cMyc. Gene expression was assessed using XP-PCR before transfection (0) and at 24, 48, 72 and 96 hours post transfection. **Blue:** 5 transcription factor. **Red:** GFP DNA only. Note the up-regulation of Gene A (cRET) and B (Brachyury).

sequences for five transcription factors (Oct4, Sox2, cMyc, Klf4 and Nanog). Expression of a complete set of endogenous, pluripotent marker genes was detected at day 30, strongly implying that these cells were de-differentiated. Further studies are underway to determine if they were truly reprogrammed to the ESC-state. A non-pluripotent stem cell line, derived from retinal pigment epithelial (RPE) cells, grew in hESC-like colonies by expressing only Nanog, cMyc and Klf4. Re-infection of this intermediate RPE cell line with the missing transcription factors (Oct4 and Sox2), however, was not sufficient to induce pluripotency.

Recent work demonstrated that only Oct4 and Sox2 were essential for the reprogramming of embryonic and adult fibroblasts<sup>11</sup> and other factors, such as cMyc, Klf4, Nanog and Lin28, serve to complement and enhance the efficiency of colony formation.<sup>4,11</sup>

A major obstacle to the use of iPS cells in regenerative therapy is the retroviral vectors used to induce pluripotency. These genome-integrating viruses have the potential to activate proto-oncogenes and generate tumors. In this study, we demonstrate the first steps toward a technology of a non-viral, particle-based

gene delivery mechanism with the aim of reprogramming human somatic cells. Single walled carbon nanotubes (SWNT) were used to transfer a mixture of the five transcription factor cDNA plasmids into primary HT cells stably transfected with a Nanog-RFP reporter construct (HT42 NP). The transfected HT42 NP cells demonstrated transient colony formation, endogenous Nanog activation and expression of two suspected indicators of reprogramming, cRET and Brachyury. It is hypothesized that HT cells may have either an epigenetic genomic plasticity, or intrinsic controls, that account for their apparent ease of transient colony formation, Nanog activation and marker gene expression. The transient expression profile of HEFs was determined after introduction of the five transcription factor plasmid cDNAs using PEI particles. Here the same suspected marker genes of reprogramming were transiently upregulated, while no colony formation could be observed within 30 days. Transduction can lead to a heterogeneous population of cells with various plasmid copy number and cells that receive some, but not all the transcription factors. This may account for the uncoordinated timing and expression levels for the five co-transduced genes, resulting in only a few reprogrammable cells.

While the overall efficiency of gene transduction was low, these studies indicate that somatic cells can be effectively reprogrammed to express pluripotency markers with retroviral gene delivery methods. It is also possible to observe minute changes in gene expression after a transient transfection protocol using SWNT. Although there is still much to be done in order to effectively reprogram adult somatic cells for application in the clinical environment, this work demonstrates that the expectation of generating iPS cells for therapeutic purposes is well on its way.

## Materials and Methods

*Lentivirus transduction:* Lentivirus production was performed as described.<sup>12</sup> Retinal pigment epithelial cells (RPE) and human embryonic fibroblasts (HEF) were infected with lentiviral particles containing the cDNA of Oct4, Sox2, Klf4, cMyc and Nanog at an approximate MOI of 10. Infected cells were grown under normal

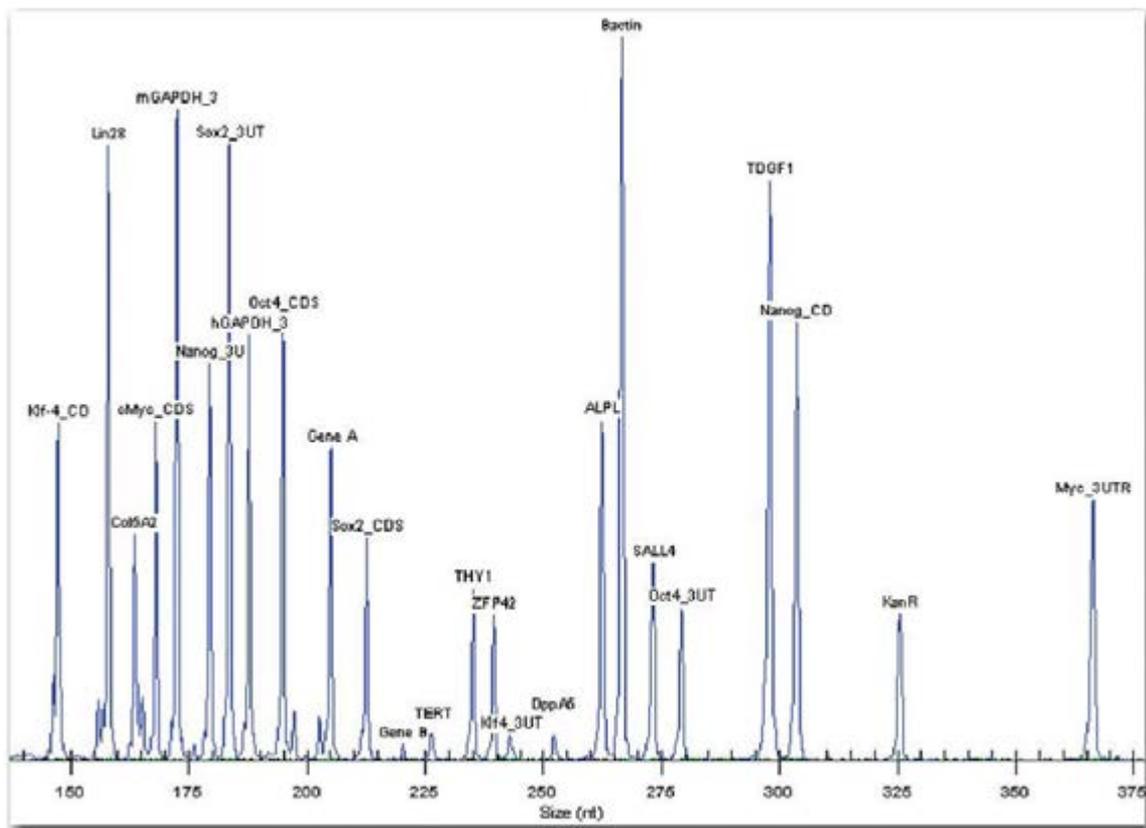
conditions in untreated dishes for six days and subsequently seeded onto mouse embryonic fibroblasts (MEF) feeder cells in hESC medium at a density of  $5 \times 10^4$  per 10-cm dish. Colonies were picked and clonally expanded with passaging every 3-7 days, onto fresh MEF feeder cells by either trypsinization (RPE) or manual picking and eventual passing using collagenase IV (HEF).

*Cell surface marker staining:* Rabbit-anti-human Tra1-60, Tra1-81 (Chemicon<sup>d</sup>) and SSEA4 (eBioscience<sup>a</sup>) antibodies were incubated with live cells for one hour in normal growth medium followed by washing and detection of the primary antibody with a secondary, TRITC-labeled sheep-anti-rabbit antibody.

*Gene expression profiling:* Forward and reverse primers for a 25-gene multiplex (Figure 8) were constructed using eXpress Profiler software (Beckman Coulter). Separate primers were designed to the coding sequence (CDS) and the 3' untranslated regions (3'UTR) of Oct4, Sox2, Nanog, Klf4, cMyc of the same mRNAs to distinguish between endogenous plus exogenous expression (CDS) and endogenous expression only (3'UTR). Additional primer sets were designed for Lin28, Col5A2, mouse GAPDH (to test for feeder layer contamination), human GAPDH, Gene A, Gene B, TERT, Thy1, Rex1 (aka ZFP42), Dppa5, ALPL, beta-Actin, Sall4 and Cripto (aka TDGF1). Primers for the internal control Kanamycin resistance gene (KanR) was also included in the multiplex design. The multiplex reverse transcription-PCR reaction for express profiling (XP-PCR) was performed according to the manufacturer's instructions using the GenomeLab™ GeXP Start Kit (Beckman Coulter), separated by capillary electrophoresis and analyzed in the GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter, Figure 8). Gene expression data was normalized to the internal control gene (KanR), quantified using a standard curve of hESC RNA ranging from 1ng to 500ng, and evaluated with GenomeLab™ GeXP Quant Tool software (Beckman Coulter) using hGAPDH and beta-Actin as normalization genes.

Gene Name	Fragment Size (nucleotides)
Klf-4_CDS	147
Lin28	158
Col5A2	164
c-Myc_CDS	168
mGAPDH_3'UTR	172
Nanog_3'UTR	179
Sox-2_3'UTR	184
hGAPDH_3'UTR	188
Oct-4_CDS	195
Gene A	205
Sox-2_CDS	213
Gene B	220
TERT	226

Gene Name	Fragment Size (nucleotides)
THY1	235
RexX 1 (ZFP42)	240
Klf-4_3'UTR	243
DPPA5	252
ALPL	262
ActinB	266
SALL4	273
Oct-4_3'UTR	279
Cripto (TDGF1)	298
Nanog_CDS	304
Kan(r)	325
c-Myc-3'UTR	366



**Figure 8. Multiplex gene list and electropherogram.** Primer sets for twenty-five gene targets (see chart at top) were assembled for XP-PCR reactions, and were then separated by capillary electrophoresis (see electropherogram). Each peak represents the PCR product of a gene. The area under each gene peak is used to quantitate the expression of that gene in each sample.

**DNA fingerprinting:** Fingerprinting was done by Cell Line Genetics Inc.\* Both of the derived cell lines (RPE and HEF iPS-like) are being grown continuously and have been confirmed to be identical to the original cell line by DNA fingerprinting.

**HT42 NP cells:** Human testicular (HT) cells were prepared as described (PCT/US2005/047437; WO2006/074075). In short, HT42 fibroblastic cells from adult human testicular tissue were recovered after enzymatic digestion and selection from adherent cells. RFP reporter cells (HT42 NP) were constructed by transducing HT42 cells with a lentivirus containing a Nanog promoter driving the expression red fluorescent protein (RFP).

**SWNT binding and treatment:** DNA binding was performed as described.<sup>10,13,14</sup> SWNT were diluted to a final concentration of 1 µg/mL and added to 1 x 10<sup>6</sup> HT target cells followed by incubation for two hours in the presence of 100 mM Chloroquine. Cells were washed and incubated with normal growth medium overnight. Cells were then trypsinized and plated onto MEF feeder cells at a density of 1 x 10<sup>5</sup> cells per 10 cm-dish and cultivated in hESC medium.

**jetPEI™\* transfection:** PEI transfection was performed according to the manufacturer's instructions (Polyplus-transfection, Inc.).

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